

Exercise 4 Quorum sensing and quorum sensing inhibition

Introduction

Many Gram negative bacteria, including *Pseudomonas aeruginosa*, *Pseudomonas aureofaciens*, *Serratia liquefaciens*, *Agrobacterium tumefaciens*, and *Erwinia carotororova* are able to regulate gene expression in accordance with population density in a process termed quorum sensing (QS). These organisms utilize N-acyl homoserine lactones (AHLs) as signal molecules (Fig. 1). During growth the AHLs accumulate, and when a certain threshold concentration is reached, target genes of the QS systems are activated (Fig. 1). The core of a QS system consists of two genes; an I gene encoding the AHL synthase and an R gene encoding a protein able to bind AHL and DNA. The N-terminal part of the R protein binds AHL and the C-terminal parts bind DNA. Most, but not all known R proteins act as transcriptional activators upon AHL binding. Some R proteins are repressors which are derepressed upon AHL binding. Most R proteins can be activated by a range of AHLs, but the cognate AHL usually has the highest affinity. The AHL signal molecule consists of two parts, a homoserine lactone ring and a fatty acyl side chain. The AHL molecules differ in the length of the side chain, which can be either unsaturated or saturated, and contains either a hydroxyl or an oxo group at the third carbon. Most AHLs are believed to diffuse across the bacterial cell wall, with the exception of long-chain AHLs, such as the oxo-C12 AHL produced by *P. aeruginosa*, that utilize an efflux pump for translocation across the cell membrane.

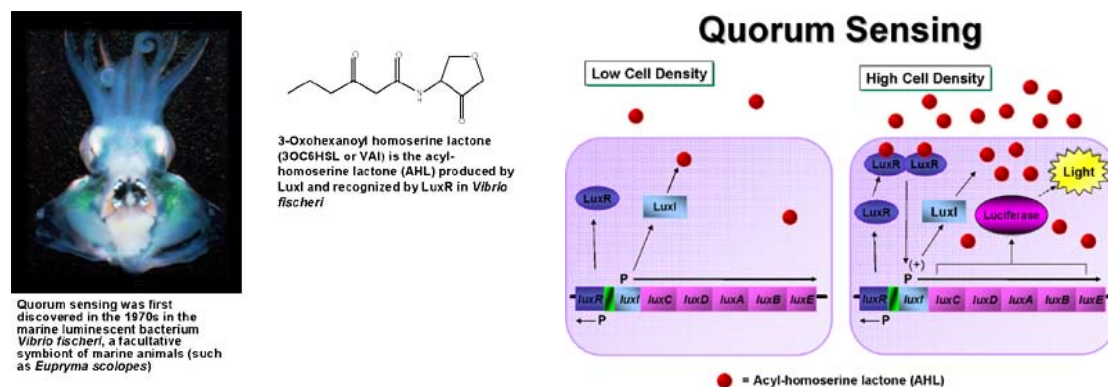


Figure 1. Quorum sensing signal molecule and activation mechanism from *Vibrio fischeri*. AHL concentration increase when the cell density is high. This results in a shift of the LuxR equilibrium towards its AHL-bound, active state. AHL binding leads to dimerization of LuxR and binding to the lux box, a 20-base pair inverted repeat located in the Plux promoter.

QS-controlled genes often encode virulence factors and gene products required for bacteria–host interactions. In addition, there is growing evidence that QS influences more complex behavioural processes such as the ability to form surface-associated structured consortia referred to as biofilms. Biofilm formation plays an important role in bacterial pathogenesis and is a common cause of persistent infections. Bacteria in biofilms are resistant to disinfectants, antibiotics and the action of host immune defences. It should therefore be possible to attenuate bacterial pathogenesis by interfering with bacterial QS systems. Knowledge about the molecular mechanisms involved in QS is important since it may form the basis for the development of new therapeutic strategies against infections.

In the present exercise we will learn how to investigate bacterial QS systems through modern molecular biology techniques. We will use bioassay systems to monitor extracellular QS AHL molecules as well as screen for QS inhibitors which can block QS process. In the molecular biology part, we will use the non-pathogenic *Pseudomonas aureofaciens* as our model strain since the exercise-lab is not classified to do molecular genetics in opportunistic pathogens. The techniques used are of course the same as those used in investigations of real pathogens. In the QS inhibitor screening part, we will use *P. aeruginosa* as our model strain.

Here is some background of *P. aureofaciens*.

P. aureofaciens produce proteases, siderophores, chitinases, HCN, phenazines and other extracellular products that are believed to account for its ecological competitive properties. In *P. aureofaciens* 30-84, the synthesis of these antimicrobial metabolites is controlled by a complex hierarchical cascade, which includes the *gacA/gacS* (a two component signal transduction system) regulon, and two QS circuits encoded by the *phzR/phzI* and the *csaR/csaI* regulons. *PhzR/phzI* regulates the production of the antibiotics phenazine-1-carboxylic acid, and 2-hydroxy-phenazine (Fig. 2), whereas *csaR/csaI*



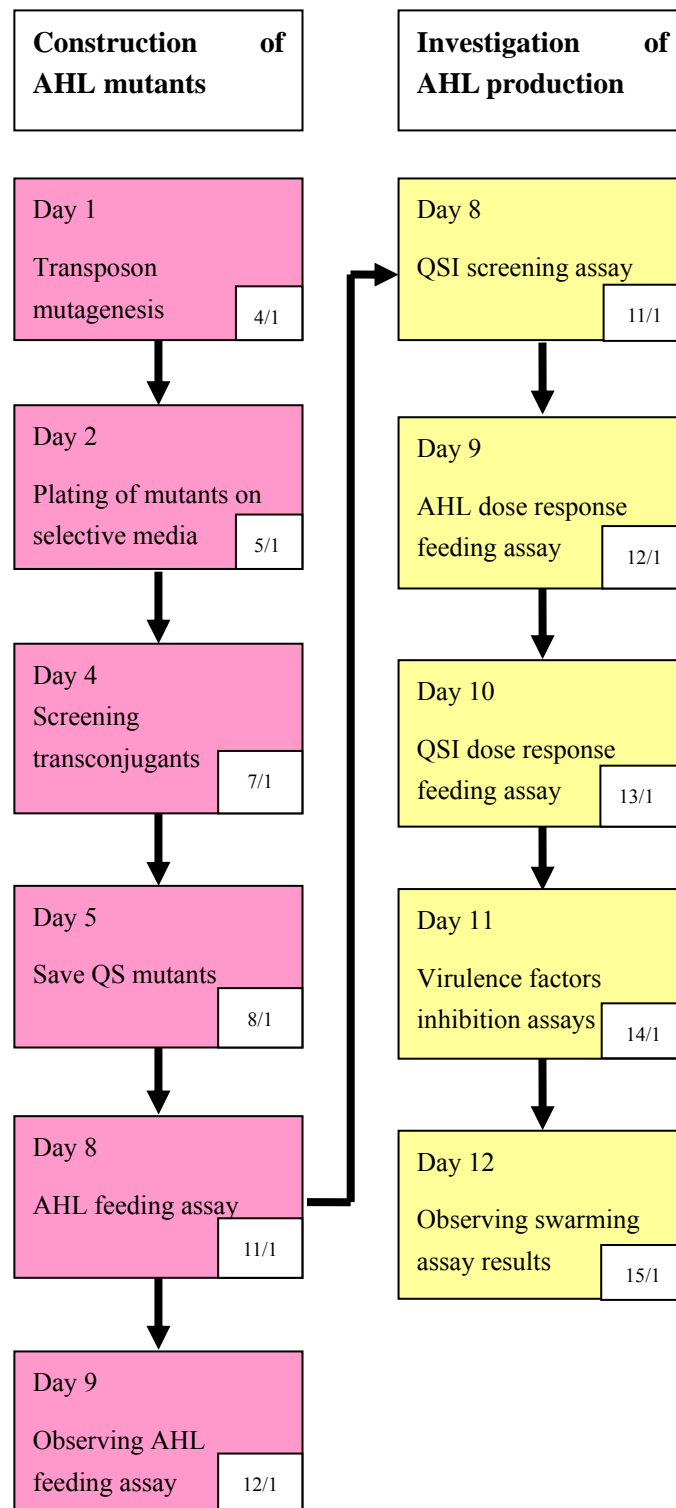
Figure 2. Phenazine in *P. aureofaciens* colony on plate

regulates currently unknown aspects of the cell surface. Mutations in either *gacS* or *gacA* dramatically reduce production of many antimicrobial metabolites as well as production of acyl-homoserine-lactone signal molecules (synthesized by *phzI* and *csaI* gene products), implying that the *gacA/gacS* regulon is above the *phzR/phzI* and the *csaR/csaI* regulon in the hierarchical cascade. Mutations which result in QS defects in *P. aureofaciens* therefore include those in *gacA/gacS*, *phzR/phzI*, and *csaR/csaI*.

Aims

In the present exercise you will use miniTn5 transposon mutagenesis to generate a *P. aureofaciens* mutant library, and you will screen for mutants with altered AHL profile through extracellular protease activity assay. The AHL profiles of the *P. aureofaciens* wild type and potential QS mutants will be characterized by the use of microtiter based bioassays. Another aim of the exercise is to isolate novel QS inhibitors by the use of special bioassays, and to study the effect of these QS inhibitors on *P. aeruginosa* virulence factor expression

Overview of experimental procedures in Exercise 4



Experimental procedures

Strains for Exercise 5

For quorum sensing part (**partI**):

Donor: MH379 (*E. coli* S17- λ pir / pUTminiTn5-Gm), Ap^R, Gm^R

Helper: SM1279 (*E. coli* HB101/pRK600), Cm^R

Recipient: JB979 (GFP tagged *P. aureofaciens* ATCC13985, *wt*), Km^R, Cm^R

AHL sensors: CV026 (*Chromobacterium violaceum*, *I*-mutant), Km^R

MH281 (*Agrobacterium tumefaciens*, pDSK519-*traR*, *traG::lacZ*), Gm^R

JB525 (MT102 / pJBA132 (*luxR*-*P_{luxR}*-*P_{luxI}*-*gfp*(ASV))), Tc^R

Ref. strains: JB977 (GFP tagged *afmI*(*csaI*) mutant of *P. aureofaciens* ATCC13985), Km^R, Cam^R

JB975 (GFP tagged *gacS* mutant of *P. aureofaciens* ATCC13985), Km^R, Cam^R

For quorum sensing inhibition part (**partII**):

QSI1: *E. coli lacO^c* / pUC18Not-*luxR*-*P_{luxI}*::*phlA*, Ap^{R-100γ}

CVO26: *Chromobacterium violaceum*, AHL⁻, Km^{R-50γ}

MH155: *E. coli* /pUCP22NotI-*P_{lasB}*::*gfp*(ASV) *P_{lac}*::*lasR*, Gm^{R-20γ}.

JB357: *E. coli* /pUC18NotI-*P_{luxI}*::*gfp*(ASV) *P_{luxR}*::*luxR*, Ap^{R-100γ}.

Part I Quorum sensing

Day 1: (Monday) Transposon mutagenesis - Triparental matings.

Materials

Over night culture of JB979

Over night culture of MH379

Over night culture of SM1279

3x 2 mL Eppendorph tubes

3x 10 mL 0.9 % NaCl for wash

2x LB plates (**Dry plates at 37°C for 30-45 min before use – This procedure is used every time**)

1. Harvest 2 mL over night culture of JB979 in a 2 mL Eppendorph tube by centrifuging for 2 minutes at 7,000g. Harvest another 2 mL JB979 in the same tube so you end up with cell mass from 4 mL cell culture in one tube.
2. Harvest 2 mL over night culture of MH379 in a 2 mL Eppendorph tube (2 min, 7,000g). Harvest another 2 mL MH379 in the same tube.

3. Harvest 2 mL over night culture of Sm1279 in a 2 mL Eppendorph tube (2 min, 7,000g). Harvest another 2 mL SM1279 in the same tube.
4. Wash the pellets twice with 1 mL 0.9 % NaCl (for removal of antibiotics)
5. Resuspend in 1 mL 0.9 % NaCl.
6. For the conjugation, mix 0.5 mL MH379, 0.5 mL SM1279 and 1 mL JB979 and spin down the cells by centrifugation (2 min., 7,000g).
7. Resuspend the pellet in 400 μ L LB and spot 4 x 100 μ L of the suspension onto 2 dry LB-plates (2 conjugation spots/plate).
8. Incubate the plates over night at **37°C (IMPORTANT)**.

The background of transposon mutagenesis (Fig. 3).

Mutants are produced in the following way. *E. coli* carrying a plasmid that contains a transposon, a transposase, a lambda-pir-dependent origin of replication and an antibiotic selection marker are mated with wild type *P. aureofaciens* JB979. Because JB979 is lambda pir-, the transferred transposon plasmid is not replicated in JB979. JB979 transposants are selected on media containing antibiotics that select for the presence of the transposon and that select against *E. coli*.

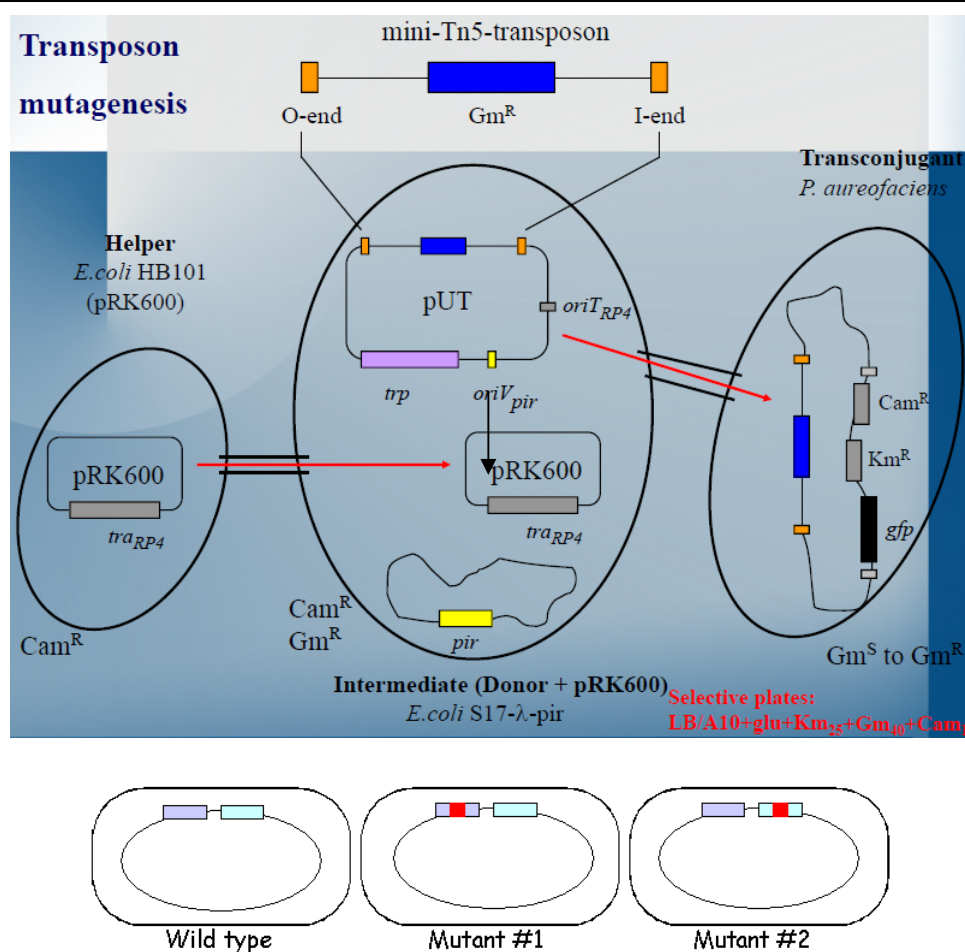


Figure 3. Transposon mutagenesis.

The background of Identification of Mutated Gene (Fig. 4).

The genomic DNA sequence adjacent to the transposon in each insertion mutant identifies the gene disrupted by the transposon. To determine the sequence of the adjacent DNA, two rounds of PCR are conducted. In the first round, a 5' primer specific to the transposon and a 3' ARB1 primer which contains a stretch of random nucleotides and a stretch of invariable nucleotides, are used to amplify genomic sequences. To enrich for genomic sequences adjacent to the transposon, a second round of PCR is performed using a nested 5' primer specific to the transposon sequence and a 3' ARB2 primer that anneals specifically with the invariant sequence present in the ARB1 primer. After cleaning up the ARB2 PCR reaction, another transposon-specific primer is used to sequence the Arbitrary PCR products. Subsequent bioinformatic analysis of the resulting sequences identifies the genomic locus adjacent to the transposon insertion.

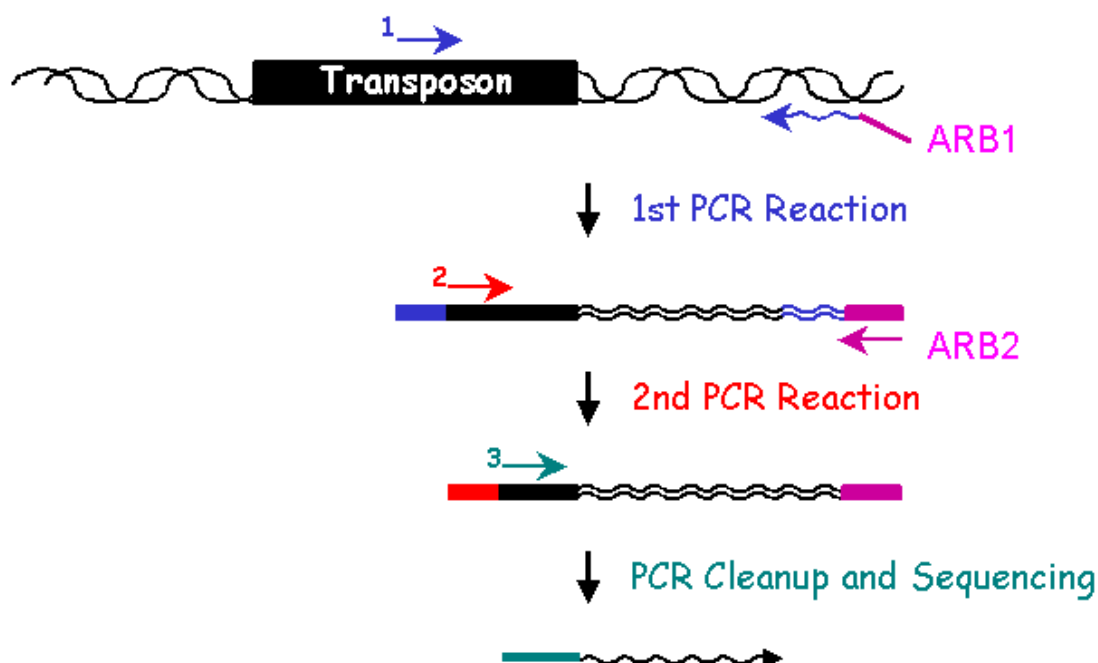


Figure 4. Arbitrary PCR Methodology: PCR Reaction 1 with primers 1 and ARB1 amplifies genomic DNA adjacent to the transposon. PCR Reaction 2 with nested primer 2 and ARB2 specifically enriches for the desired products from PCR Reaction 1. Products from PCR Reaction 2 are sequenced directly using primer 3.

Day 2: (Tuesday) Plating of transconjugants on selective plates.

Materials

1x 2 mL Eppendorf tube

2x 10 mL 0.9% NaCl

2x LB Km25-Gm40-Cam10 plates

1. Gently scrape off the 4 conjugation spots with a 1 mL tip, use 1 mL of 0.9 % NaCl to wash the plate (avoid collecting agar)
2. Resuspend the conjugation spots together in 1 mL of 0.9 % NaCl. Spin the cells down (2 min., 10,000 g).
3. Wash the cells twice with 1 mL 0.9 % NaCl
4. Resuspend the conjugation mix in 1 mL of 0.9 % NaCl.
5. Spread 100 μ L of the washed conjugation mix **and** a ten-fold diluted conjugation mix (10 μ L mixed with 90 μ L 0.9 % NaCl) onto 2x LB Km25-Gm40-Cam10-plates (selective plates).
6. Incubate plates at 28°C
7. The remaining 890 μ L conjugation-mix is **stored** in the refrigerator for later use.

Day 4: (Thursday) Phenotypic screening of transconjugants.

Materials

Sterile tooth picks

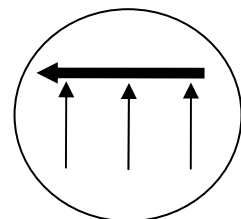
Plates with JB979, JB977, JB975, CVO26, and JB525

At least 4x ABTG + Cas-Amino Acids (0.5% glucose and 0.5% Cas-AA) plates

In order to determine the AHL phenotypes of all the isolated mutants we will initially perform cross feeding experiments on agar plates between the isolated mutants and two different AHL monitor strains called CV026 (produces a purple pigment (violacein) in the presence of C4, C6, and to some extent C8 AHLs) and JB525 (produces GFP (green fluorescent protein)) in the presence of C6, C8, C10 and C12 AHLs).

1. Inspect the colonies of the transconjugants. Pick up mutants by the following two group:
 - a. Phenazine pigment deficient mutants. Find and mark 20 white colonies (without brown color) from the transconjugant plates with a marker pen.
 - b. Smooth (mucoid) colony mutants. Find and mark 20 smooth colonies from the transconjugant plates with a marker pen.
2. Using a sterile tooth pick, cross streak the mutants of selected transconjugants against CV026 (AHL monitor strain), by forming T's on LB plates (see below). **Remember to change tooth pick after every streak.**
3. Using a sterile tooth pick, cross streak the mutants of selected transconjugants against JB525 (another AHL monitor strain), by forming T's on ABTG-CasAA-plates (see below). **Remember to change tooth pick after every streak.**

The T's are made as follows: Streak monitor strain across the upper part of the plate. Streak up the mutants from the opposite part towards the sensor strain **WITHOUT touching** the sensor strain, but **VERY CLOSE TO** (see drawing).



4. Likewise, cross-streak JB979, JB977, and JB975 against CV026 by forming T's on LB plates and against JB525 by forming T's on ABTG-CasAA-plates as controls.
5. Incubate plates over night at 28°C.

Day 5: (Friday) Inspection of AHL cross-feeding experiment.

1. Inspect the cross-feeding experiments, and note whether the potential AHL negative mutants give rise to an AHL negative phenotype (compare the impact of your mutants, JB979, and JB977 on the AHL sensor strains). Cross-feeding experiments involving CV026 are visually inspected for production of purple pigment. Cross-feeding experiments involving JB525 are inspected for production of GFP using a fluorescent microscope. All observations are noted in the result sheet.
2. Restreak quorum sensing mutants on fresh LB -Km₂₅-Gm₄₀-Cam₁₀ and incubate on your table at room temperature over weekend for the usage of the next exercises.

Please remember to bring samples next Monday for the partII of the excise quorum sensing inhibition! This sample can consist of leaves from your pot plant, fresh vegetables, herbs or herb medicines or whatever you can think of.

PartII Quorum sensing inhibition

Day 8 (Monday): Last exercise of partI and start exercise of partII.

AHL feeding assay (partI)

Since QS molecule AHL are small diffusible molecules, we are going to check whether synthetic AHLs and AHL produced from neighbor cells could restore the phenotypes of selected QS mutants to the wild-type level.

1. Synthetic AHL feeding assay for Phenazine pigment production.
Inoculate 5 selected phenazine deficient QS mutants, two control strains (JB977 and JB975) and the wild type JB979 strain in 16 ABTG-CasA tubes (inoculate two tubes (**a** and **b**) for each strain). Add 1 µM HHL (final concentration) into tube **a** of each strain except the wild type JB979. Add 1 µM BHL (final concentration) into tube **b** of each strain except the wild type JB979. Incubate the tubes at 28 degree over night.
2. Neighbor cells AHL feeding assay for rough colony phenotype.
Similar to the cross-feeding experiments of Day 5, using a sterile tooth pick, cross streak 5 selected smooth colony QS mutants against wild-type JB979 by forming T's on LB plates. **Remember to change tooth pick after every streak.** Then cross streak 5 selected smooth colony QS mutants against control QS mutant JB 977 by forming T's on LB plates. Then cross streak 5 selected smooth colony QS mutants against control QS mutant JB 975 by forming T's on LB plates.

Natural source library screening for QSI compounds (partII)

Materials QSI1: *E. coli lacO^c* / pUC18Not-*luxR*-P_{*luxI*}::*phlA*, Ap^{R-100γ}
CVO26: *Chromobacterium violaceum*, AHL⁻, Km^{R-50γ}

In this exercise you should use your own sample.

Sample preparation

Crush or chop your sample, transfer it to a 100 ml flask, add ≈ 2 ml ethyl acetate/g sample (do not use more than 5-10 g sample). Mix well. The sample is left in the **fumehood** for a couple of hours. Shake the flask from time to time. Filter the sample and transfer to a 100 ml beaker. Evaporate most (but not all) ethyl acetate. Make QSI indicator plates late in the afternoon, one of each type for each sample as described below. The concentrated extracts which are not used in the QSI indicator plates are transferred to Eppendorf tubes and stored at -20 °C for later use.

All work with ethyl acetate is done in the fumehood!

The screening systems

QSI1 is a genetically engineered selector system based on the *lux* quorum sensing system from *Vibrio fischeri* and contains *luxR* and P_{*luxI*}::*phlA* on a cloning vector. *phlA* encodes phospholipase A, which when expressed causes cell lysis. The expression of LuxI is under the control of the regulator protein LuxR. When the cognate signal molecule OHHL interacts with LuxR it induces the transcription of the gene under the control of the *luxI* promoter. In QSI1 this leads to the expression of phospholipase A thereby causing lysis of the cell. If a quorum sensing inhibitor is present along with OHHL the interaction between the OHHL activated LuxR protein and the *lux* box upstream the *luxI* promoter is hindered and phospholipase is not produced, hence the cell survives and is able to proliferate. When test samples with quorum sensing inhibitory activity are applied to wells in the agar containing the QSI1 selector strain along with OHHL and X-gal, a blue zone around the wells appears accompanying growth, as the selector strain is only capable of growth if quorum sensing is inhibited. Toxic compounds present in the test sample give a clearing zone around the well where growth is not possible. Compounds which both possess QSI and toxic properties give a clearing zone near the well, where the highest concentration is present, followed by a blue zone indicating the QSI activity.

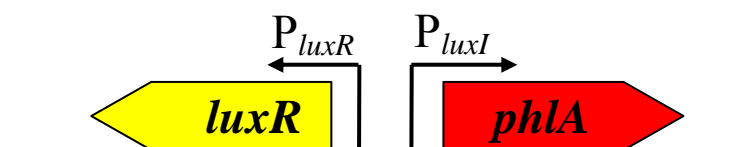


Figure 5. Schematic outline of the essential parts of the plasmid in QSI1

CVO26 *Chromobacterium violaceum* is a Gram-negative bacterium commonly found in soil and water, which produces the characteristic purple pigment violacein. CVO26 is a pigment deficient mini-Tn5 mutant of *C. violaceum* in which pigment production can be restored by addition of *N*-hexanoyl-L-homoserine lactone (HHL). However, if a compound with quorum sensing inhibitory properties is added along with HHL, the production of violacein is abolished. Hence this biosensor can be used when screening for quorum sensing inhibitors, since a colourless zone around the well where the test sample has been applied, indicates the presence of an inhibitor.

Materials for two QSI1 indicator plates: 45 ml melted (45 °C) BT-agar, 5 ml A10, 1 ml glucose 20 %, 250 µl cas-amino acids 20 %, 20 µl 0.1 mM OHHL (use the 10 mM OHHL and make 100 µl of a 100-fold dilution), 10 µl Ap 500 mg/ml, 25 µl X-gal 40 mg/ml, 200 µl o.n. culture of QSI1.

Materials for two CV026 indicator plates: 50 ml melted (45°C) LB-agar, 200 µl o.n. culture of CV026, 50 µl kanamycin, 10 µl 10 mM HHL

Prepare two of each type of indicator plates. After the two sets of plates have solidified, punch a hole for each of your samples (max 5 samples pr. plate, if you have more samples then prepare additional plates) in the agar of each plate using the butt end of a 1000 µl pipette tip. Add 100 µl of your extract to the well in a CVO26 plate and 50 µl extract to a QSI1 plate. Do the same with another set of plates with a known quorum sensing inhibitor delivered to you. Plates are incubated 30°C (plates are NOT incubated upside-down!). Store the remaining extracts, HHL and OHHL, antibiotics and X-gal at -20°C. Store remaining glucose and cas-amino acids in the fridge (4°C). Remember to write your names/team number on them.

Day 9 (Tuesday): Last exercise of partI and start exercise of partII.

Observation of the AHL feeding assay (continuation of partI)

Check the phenazine pigment production and the cell morphology of the two AHL feeding assays and remember to take pictures for the report.

Dose-response investigation and optimization of AHL concentration

Strains

MH155: *E. coli* /pUCP22NotI- $P_{lasB}::gfp(ASV)$ $P_{lac}::lasR$, Gm^{R-20γ}.

JB357: *E. coli* /pUC18NotI- $P_{luxI}::gfp(ASV)$ $P_{luxR}::luxR$, Ap^{R-100γ}.

The AHL monitor systems mentioned above are bacterial strains genetically engineered to give a “visible” response if signal molecules are present in the growth medium. Before the AHL monitor systems are employed their limits and specificity have to be determined. In this exercise detection limit and saturation point are found for two AHL monitor strains (Table 1) and four different AHLs (Table 2). The QS systems involved are the *las* system from *P. aeruginosa* and the *lux* system from *Vibrio fischeri*. Each quorum sensing system is represented as an AHL monitor. The monitors have the promoter of a target gene for the given system fused to the gene encoding unstable green fluorescent protein; Gfp(ASV). When the gene encoding Gfp(ASV) is fused to a promoter positively regulated by quorum sensing, the induction of the promoter increases the expression of Gfp(ASV). Hence, the elevated expression of the quorum sensing controlled gene can be measured as increased fluorescence. The addition of a quorum sensing inhibitor to this type of construct results in lowered expression of Gfp(ASV) to an extent proportional to its efficiency as quorum sensing inhibitor. A toxic compound would also lower the fluorescence, but by measuring the growth rate along with the fluorescence this can be accounted for. The two fusions are located on plasmids harboured by *E. coli* (as outlined in Fig. 6).

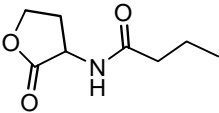
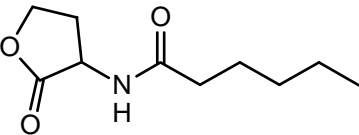
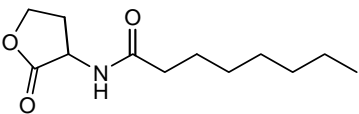
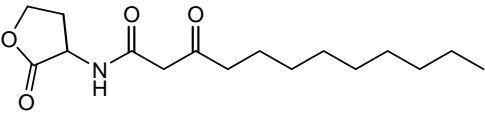
Table 1. The two AHL monitors employed.

Monitor	QS system	Target gene	Reporter gene
MH155	<i>las</i>	<i>lasB</i>	<i>gfp(ASV)</i>
JB357	<i>lux</i>	<i>luxI</i>	<i>gfp(ASV)</i>



Figure 6. Schematic of the essential parts of a MH155. b and JB357.

Table 2. The four AHLs employed.

AHL	Abbreviation	Structure
N-Butanoyl-L-HSL	BHL	
N-hexanoyl-DL-HSL	HHL	
N-octanoyl-DL-HSL	OHL	
N-(3-oxo)-dodecanoyl-L-HSL	OdDHL	

Assay for determination of dose-response curves for the two monitor strains and different AHLs

We are going to use 1:2 serial dilutions in this assay.

"Serial dilution" - This term is frequently used and refers to a "multiple" dilution problem. In other words, an initial dilution is made and then this dilution is used to make a second dilution, and so on. For example, a 1:2 serial dilution is made using a 150 µl volume of sample. This expression indicates that 150 µl of sample is added to 150 µl of H₂O and then mixed. This initial dilution is 1:2. Then, 1 mL of this dilution is added to 150 µl of H₂O further diluting the sample. This same process is continued (Fig. 7).

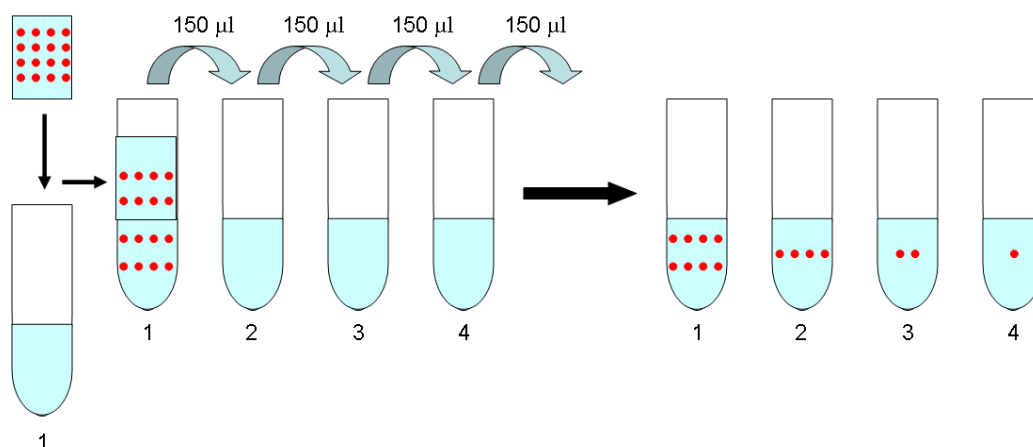


Figure 7. Illustration of 1:2 serial dilution method.

- Mix 90 ml BT, 10 ml A10, 2.5 ml 20% glucose and 2.5 ml 20 % Cas-amino acids.
- Add 150 µl media to all 96 wells of a (“black”) microtiter plate using a multipipette.
- Add 130 µl additional media to the wells in the first column (column 1)
- Add 20 µl of signal molecule to the wells in the first column; BHL in row A+E, OHHL in row B+F, OHL in row C+G and OddHL in row D+H.
- Make **1:2 serial dilutions** in columns 1-11 by transferring 150 µl from column 1 to column 2 and so on. **The excess 150 µl from column 11 is discarded.** Column 12 is left as reference, that is, AHLs are not added to the wells in this column.
- Mix 4 ml o.n. monitor strain and 11 ml medium in a 15 ml tube (do this for both monitor strains).
- Add 150 µl monitor strain from the 15 ml tube to each well. JB357 is added to all wells in row A-D, and MH155 is added to the wells in row E-H
- Put on a lid and incubate 3-4 hours at 37 °C.
- Measure OD and fluorescence using the multi label plate reader.

Again; store the remaining signal molecules (BHL, OHHL, OHL and OddHL) at -20°C. Store remaining glucose and cas-amino acids in the fridge (4°C).

Data analyses

Draw dose-response curves for the two monitor strains showing relative fluorescence (fluorescence pr. OD) as a function of the concentration of the different signal molecules. Also find the relation between the fluorescence and the concentration of the different signal molecules as well as the relationship between OD and the concentration of the different signal molecules.

Characterize the two monitors (i.e. broad/narrow spectrum, cognate AHLs, concentration range etc.)

Do the data analysis as soon as possible. You need the results for day 11's experimental work!!!

Day 10: (Wednesday) Dose-response curves with quorum sensing inhibitors

This part is focused on creating a dose-response curve in the presence of quorum sensing inhibitors. You will use the optimal type of AHL and AHL concentration determined by the dose-response optimisation. Use the AHL concentration giving about 75 % of maximal induction of fluorescence (if your experiments went wrong, use a final concentration of 100 nM of the signal molecule giving the highest relative fluorescence). Use patulin, 4-nitropyridine-N-oxide, and penicillic acid as QSI compounds. Also test the extract(s) you made for quorum sensing inhibitory properties.

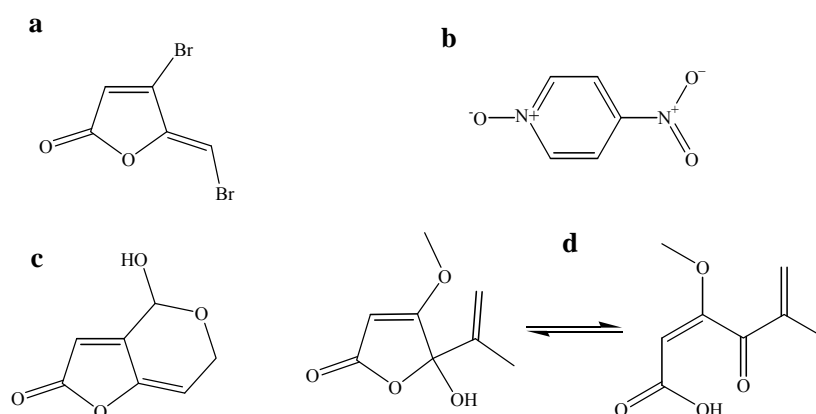


Figure 8. Quorum sensing inhibitory compounds **a.** the halogenated furanone C30, **b.** 4-nitropyridine-N-oxide **c.** patulin **d.** penicillic acid.

Assay for determination of dose-response curves in the presence of QSIs

- Mix 90 ml BT, 10 ml A10, 2.5 ml 20% glucose and 2.5 ml 20 % Cas-amino acids.
- Add 150 μ l media to all 96 wells of a (“black”) microtiter plate using a multi-pipette.
- Add 130 μ l additional media to the wells in the first column (column 1)
- Add 20 μ l of QSI compound to the wells in the first column; patulin in row A+E, 4-nitropyridine-N-oxide in row B+F, penicillic acid in row C+G and your own extract in (in row D+H).
- Make **1:2 serial dilutions** in columns 1-11 by transferring 150 μ l from column1 to column 2 and so on. **The excess 150 μ l from column 11 is discarded.** Column 12 is left as reference, that is, QSIs are not added to the wells in this column.
- Mix 4 ml o.n. monitor strain and 11 ml medium in a 15 ml tube and store for later use (do this for both monitor strains).

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- Add the AHL you found to be optimal to the 15 ml tube with monitor strain in a concentration being the double of the concentration which was capable of inducing the fluorescence to 75 % of the maximal value
- Add 150 µl monitor strain from the 15 ml tube to each well. JB357 is added to all wells in row A-D, and MH155 is added to all the wells in row E-H
- Put on a lid and incubate 3-4 hours at 37 °C
- Measure OD and fluorescence in the multi-label plate reader.

Data analyses

Draw dose-response curves for the two monitors showing fluorescence (and OD) as function of concentration of the quorum sensing inhibitors. Find the highest concentration of each inhibitor, which does not have an impact on growth (i.e. OD is not affected compared to the reference). **Please write down and remember these concentrations, which are going to be used for the later excises.**

Inoculation for virulence factor assays (Important!)

Inoculate three 10 ml LB tubes using 5 µl of a PAO1 culture. To one tube 20 µl patulin is added and to another 20 µl penicillic acid is added. The third serves as an untreated control. Take a 4th tube with LB for reference, nothing is added to this tube but it is incubated at 37 °C along with the other tubes. Remember to write team number and content on all tubes.

Day 11: (Thursday) Quorum-sensing regulated virulence factor inhibition assays.

Swarming is a rapid mode of surface translocation. The bacterium *Serratia liquefaciens* MG1 is able to swarm on top of 0.6% agar plates. This process is dependent not only on synthesis and operation of flagella, but also on production of a surface active compound – the surfactant serrawettin W2. The gene encoding serrawettin synthase, SwrA, is controlled by the *swr* quorum sensing system. If the quorum sensing system is inhibited, *S. liquefaciens* is unable to swarm. This can be employed as a screening for quorum sensing inhibitors. Here, two quorum sensing inhibitors, the halogenated furanone patulin and penicillic acid (Fig. 7) are tested for inhibition of QS controlled surface motility.

Swarming assay

- Add 4 ml melted swarm medium (ABTG-casa with 0.6% agar) to each well of two 6 well tissue culture test plates.
- Quickly afterwards, add the test compounds as indicated in the table below (stock solution of patulin and penicillic acid is 10 mM). Use one test plate for each compound. If your own extract showed quorum sensing inhibitory properties use this instead of penicillic acid, in non-growth-inhibitory concentrations (refer to your results obtained in the *Dose-response curves with quorum sensing inhibitors* part of this exercise).

Well #	1	2	3	4	5	6
Test comp. (µl)	40	20	4	2	1	0
Final conc. (µM)	100	50	10	5	2,5	0

- Gently shake the plate and/or mix each well with a sterile 1000 µl tip.
- After the agar has solidified, the plates are left to dry 20 minutes without lid. Take measures to avoid contamination of the plates.
- Make a 10⁴ dilution of an o.n. culture of *Serratia liquefaciens* MG1.
- Add 5 µl of the the diluted o.n. culture to one “end” of each well.
- Put on lids and incubate over night at 30°C.

Rhamnolipid emulsification activity assay.

Rhamnolipid is a biosurfactant, do you have any ideas how this compound will harm host issues during the infections? On the other hand, do you have any ideas how this compound can be applied in the industry and pollution controlling?

- Shake the tubes gently for 10 seconds and then transfer 1.5 ml overnight culture of the three inoculated tubes from Day 12 to three new centrifuge tubes separately.
- Centrifuge at 10, 000 rpm for 2 min.
- Transfer 1 ml supernatant from the three centrifuge tubes to three 10 ml yellow cap tubes. **Transfer the left 0.5 ml supernatant from the three centrifuge tubes to three new 2 ml centrifuge tubes for the next part of the exercise.**
- Add 1 ml n-hexadecane to these three tubes separately.
- Screw the yellow caps and vortex the 10 ml yellow cap tubes for 2 min.
- Allow these yellow cap tubes to stand on the table for 1 hour before inspection.

Emulsification activity was defined as the height of the emulsion layer divided by the total height and expressed as a percentage.

Blue pigment pyocyanine assay.

Pyocyanine (or pyocyanin) is an antibiotic pigment produced by the *P. aeruginosa*. It is a redox-active virulence factor which allows *P. aeruginosa* to kill cells, disrupts cilia actions, inhibit lymphocyte proliferation, and alter phagocytic function. Due to its redox-active properties, pyocyanin generates reactive oxygen species that induce oxidative stress in bacteria and mammalian cells.

(NB: work in the fumehood since we are going to work with chloroform!)

- Take the three centrifuge tubes with 0.5 ml supernatant of the overnight cultures from the emulsification activity assay to the fumehood.
- Add 1 ml chloroform to the three tubes separately.
- Close the caps of the three tubes and shake for 30 seconds (here chloroform will extract the pyocyanine to the organic phase).
- Transfer the 1 ml chloroform organic phase from the three tubes to three new centrifuge tubes separately.
- Add 1 ml 0.2 N HCl to the three new centrifuge tubes with chloroform.
- Close the caps of the three tubes and shake for 30 seconds (here HCl will extract the pyocyanine to the aqueous phase).
- Transfer 1 ml of the three aqueous phase pyocyanine extractions to three cuvetts separately and measure 520 nm at spectrophotometer.

Day 12: (Friday) Observe swarming assay results

Questions

How to measure and identify QS signal molecules ?

What is the role for QS regulation in environment and hosts?

How to develop a QS inhibitor into a drug?